

REMARKS

Favorable reconsideration is respectfully requested in view of the foregoing amendments and the following remarks.

Applicants sincerely thank the Examiner and his supervisor for holding a personal interview with Applicants' representative on September 10, 2008. The Examiner and his supervisor's kind suggestions have been incorporated into this Response.

I. CLAIM STATUS AND AMENDMENTS

Claims 1-6, 11-21 and 26 were pending in this application when last examined and stand rejected.

Claims 8 and 12 are cancelled without prejudice or disclaimer thereto.

Claim 1 is amended to reduce the claimed genus of cobalamin derivatives in order to expedite prosecution and without acquiescence to the correctness of the Office's rejections. Further, claim 1 is amended to include limitations from claims 12 and 15.

Claims 13, 15, 17 and 18 are amended to correct informalities.

Claims 20-21 are amended in order to expedite prosecution and without acquiescence to the correctness of the Office's rejections.

No new matter has been added.

II. ENABLEMENT REJECTION

On pages 2-4 of the Office Action, claims 20-21 were rejected under 35 U.S.C. § 112, first paragraph, for failing to comply with the enablement requirement. This rejection is overcome, as applied to amended claims 20-21, for reasons which are self-evident.

III. OBVIOUSNESS REJECTION

On pages 5-7 of the Office Action, claims 1-6, 8, 11-21 and 26 were rejected under 35 U.S.C. 103(a) as obvious over Morgan et al. (WO 95/27723) in view of Grissom et al. (U.S. 6,797,521) and Collins (U.S. 5,739,313).

Applicants respectfully traverse this rejection as applied to the amended claims.

Applicants note that claim 1 has been amended to significantly reduce the genus of cobalamin derivatives. For instance, R^b is now the only spacer-chelator group and such is

defined as consisting of an aliphatic chain of 2-4 carbon atoms carrying the chelators shown in Formulas (II) to (IX).

Further, Applicants note that this claim requires that the claimed cobalamin derivatives have no binding affinity or less than 20% binding affinity to TC II and retain activity as a vitamin B12 substitute.

Such genus with the claimed attributes is not obvious over Morgan et al. in view of Grissom et al. and Collins to a person of skill in the art.

Authors and inventors of prior art publications describing vitamin B12 derivatives for use in diagnosing and treating neoplastic diseases did either not know the importance of TC II affinity (or rather lack of TC II affinity), or misjudged the importance of TC II affinity because it was generally felt that TC II binding is important to transport vitamin B12 and vitamin B12 derivatives to the site of action within neoplastic cells. A summary of the knowledge at the time of filing the invention with respect to the transcobalamins (TC I, TC II and TC III, also called R-binder proteins or haptocorrins) is found on page 2 and 3 of the instant patent application.

The present inventors not only found an unexpected way forward in the diagnosis and treatment of neoplastic diseases by choosing vitamin B12 derivatives having no binding affinity or low binding affinity to TC II, but furthermore found that cobalamin derivatives have to retain vitamin B12 activity in order to be useful for the intended diagnostic and therapeutic purposes. There is no prior art or scientific information to this aspect. The inventors found for the first time that uptake of vitamin B12 derivatives in neoplastic cells is dependent on the claimed proper vitamin B12 activity.

For further illustration of the merit and accomplishments of the inventors, enclosed is a scientific paper (Cancer Research 2008, 68:2904-2911) dealing with the subject of the invention and published only recently. The inventors of the present application are also authors of this paper. The compounds illustrated in the paper include Tc^{99m}-complexed vitamin B12 derivatives (see Figure 1) corresponding to the compounds of the patent application of formula (I) wherein R^b is a spacer-chelator group comprising a chelator of the formula (II); R^c, R^d, R^e and R^R are hydrogen; X is cyano; and the spacer is an aliphatic chain of 2 to 4 carbon atoms. The number of carbon atoms in the chain shows up in the compound designation Tc(2)-Cbl, Tc(3)-Cbl and Tc(4)-Cbl used in the paper.

In particular, it is noted that Tc(4)-Cbl, which is a derivative of the claimed invention, does not bind to TC II. Please see page 2907 of the enclosed reference. On the other hand, ⁵⁷Co-Cbl, ¹¹¹In-DTPA-Cbl and ^{99m}Tc-tricarbonyl labeled Tc(6)-Cbl all bind to TC II. Please also see Table 1 of the enclosed reference. As shown in Figure 3 of this reference, these cobalamin derivatives that do bind to TC II were highly distributed to nontumor tissue. On the other hand, Tc(4)-Cbl, which did not bind TC II more specifically bound to tumor tissue. As indicated on page 14, lines 19-25, of the specification, the property of the claimed derivatives for specifically concentrating in tumor tissues is highly advantageous to reduce detrimental uptake of cobalamin derivatives carrying radioactive isotopes or nonradioactive cell-destroying agents. Thus, the cited references fail to render the claimed invention obvious as they fail to teach or suggest that low or no TC II binding activity results in greater selectivity towards uptake by tumor cells and therefore results in superior B12 derivatives for treatment or diagnosis of neoplastic disease.

Further, Applicants note that Morgan teaches a large genus of cobalamin derivatives. However, even if Morgan in combination with the other cited references could be envisioned as teaching the claimed genus, such would not be obvious to a person of skill in the art because the cited references fail to teach the criticality of low or no TC II binding. Such situation is analogous to a “results effective variable” as indicated in MPEP 2144.05(2)(B). Thus, a person of skill in the art would be unable to use the teachings of the cited references to obtain the claimed genus of cobalamin derivatives since these references fail to teach the importance of low or no TC II binding.

In fact, Morgan et al. (WO 95/27723) actually teaches away from the claimed invention.

Morgan et al. (page 3, lines 15 to 33, figures 22 to 25, page 12, line 26 to page 13, line 5) point to the importance of binding transcobalamin II. Thus, a skilled artisan would not consider vitamin B12 derivatives not binding (or only slightly binding) transcobalamin II as useful for tumor diagnostic and therapeutic purposes.

Thus, this rejection is untenable and should be withdrawn.

Furthermore, the following differences are noted between the cited references and the claimed invention.

In regard to Morgan et al., the Office points to page 36, lines 20-24 describing use of radioisotopes conjugated to cobalamin. Applicants note that this text describes a vitamin B12/biotin conjugate which then may be further complexed with a biotin binding protein

conjugated to a radioisotope, representing a construct much more complicated than the one illustrated and claimed in the present invention.

Further, the Office points to pages 14 through 18 describing various linkers. Although, theoretically, these described linkers may be regarded as also including structural elements able to chelate metal ions, the description does not suggest such information to one skilled in the art. It is a hindsight interpretation that one would conclude from the cited linker description that a spacer-chelator should be selected.

The Office also points to the description of a cobalamin carrying chloroquine (figure 6, page 54, example 11). Chloroquine derivatives are not metal chelators. The skilled artisan would not be guided to modify a chloroquine derivative in order to make it a (reasonably) good metal chelator, since chloroquine is chosen as a therapeutic agent or “rerouting moiety” for concentration in lysosomes. The skilled artisan would know that the therapeutic and rerouting property is lost if the amino function in 4-position (para) relative to the quinoline nitrogen is moved into a 3-position (meta) or 8-position (peri) required for metal complexation. Metal complexes are not envisaged as rerouting moieties.

Grissom et al. (US 6,797,521) describe fluorescent cobalamins. In column 9 (specifically designated by the Office), the use of radioactively labeled cobalamin is discouraged (lines 2125). It is not seen how Grissom et al. taken alone or in combination with Morgan et al. would make the instant invention obvious. Grissom et al. are silent on TC II binding or vitamin B12 activity.

Collins (US 5,739,313) describes radionuclide labeled cobalamins that have chelators/linkers attached to them. The Office particularly points to col. 3, line 5 to col. 6, line 51. The described cobalamins are useful for imaging and targeting tumors (col. 3, lines 47-52). Applicants cite from col. 3, lines 32-43, the paragraph preceding the claimed use for imaging and targeting tumors:

These compounds were found [...] to localize within the liver, kidney, pancreas, and spleen. Therefore, the present compounds can be used to evaluate hepatic, splenic, renal, pancreatic, and small bowel function in mammals [...] detecting its presence in the target organ.

The skilled artisan would not consider these compounds particularly suitable for tumor imaging and/or targeted cancer therapy as claimed by Collins in col. 3, line 48, if at the same time these compounds accumulate in normal healthy organs. Collins demonstrates to the skilled

artisan that the cobalamin type compounds of the prior art have low selectivity. Collins actually causes the skilled artisan to look elsewhere for selective compounds not accumulating in liver, kidney, pancreas, and spleen.

Thus, for the above-noted reasons, it would not have been obvious over Morgan et al. (WO 95/27723) in view of Grissom et al. (US 6,797,521) and Collins (US 5,739,313), to make the instantly claimed cobalamin derivatives and use them in a method of treating and diagnosing neoplastic diseases.

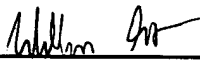
CONCLUSION

In view of the foregoing amendments and remarks, it is respectfully submitted that the present application is in condition for allowance and early notice to that effect is hereby requested.

If the Examiner has any comments or proposals for expediting prosecution, please contact the undersigned attorney at the telephone number below.

Respectfully submitted,

Hans Jorg TREICHLER et al.

By: 

William R. Schmidt, II
Registration No. 58,327
Attorney for Applicants

WRS/lc
Washington, D.C. 20006-1021
Telephone (202) 721-8200
Facsimile (202) 721-8250
September 24, 2008

ATTACHMENTS

- A. Waibel et al., "New Derivatives of Vitamin B12 Show Preferential Targeting of Tumors", *Cancer Research*, (April 15, 2008), **68**(8): 2904-2911.

New Derivatives of Vitamin B12 Show Preferential Targeting of Tumors

Robert Waibel,¹ Hansjörg Treichler,² Niklaus G. Schaefer,³ Dave R. van Staveren,¹ Stefan Mundwiler,⁴ Susanne Kunze,⁴ Martin Küenzi,⁷ Roger Alberto,⁴ Jakob Nüesch,⁷ Alexander Knuth,³ Holger Moch,⁵ Roger Schibli,^{1,6} and Pius August Schubiger^{1,6}

¹Center for Radiopharmaceutical Science, Paul Scherrer Institute, Villigen PSI, Switzerland; ²Ringackerstrasse 1, Känerkinden, Switzerland; ³Clinic of Medical Oncology, University Hospital of Zurich; ⁴Department of Inorganic Chemistry, University of Zurich; ⁵Institute of Surgical Pathology, Department Pathology, University Hospital of Zurich; and ⁶Department of Chemistry and Applied Biosciences of the Eidgenössische Technische Hochschule Zürich, Zurich, Switzerland; and ⁷Solidago AG, Bern, Switzerland

Abstract

Rapidly growing cells show an increased demand for nutrients and vitamins. The objective of our work is to exploit the supply route of vitamin B12 to deliver new derivatives of this vital vitamin to hyperproliferative cells. To date, radiolabeled (⁵⁷Co and ¹¹¹In) vitamin B12 derivatives showed labeling of tumor tissue but also undesired high accumulation of radioactivity in normal tissue. By abolishing the interaction of a tailored vitamin B12 derivative to its transport protein transcobalamin II and therefore interrupting transcobalamin II receptor and megalin mediated uptake in normal tissue, preferential accumulation of a radiolabeled vitamin in cancer tissue could be accomplished. We identified transcobalamin I on tumors as a possible new receptor for this preferential accumulation of vitamin-mediated targeting. The low systemic distribution of radioactivity and the high tumor to blood ratio opens the possibility of a more successful clinical application of vitamin B12 for imaging or therapy. [Cancer Res 2008;68(8):2904–11]

Introduction

All living cells require vitamin B12 (cobalamin) for survival. Before its use in cells, cobalamin must be metabolized into biologically active methylcobalamin or adenosylcobalamin. In humans, these cofactors are used in two enzyme systems: The first enzyme, methionine synthase, uses methylcobalamin to produce methionine from homocysteine. The second enzyme, methylmalonyl-CoA mutase, uses adenosylcobalamin as a coenzyme to enter the tricarboxylic acid cycle. Thus, it is not surprising that rapidly dividing tissues requiring methionine and thymidine for cell proliferation have an increased demand of cobalamin. Cobalamin has several characteristics that make it an attractive entity for *in vivo* tumor diagnostic and possible therapeutic application: (a) cobalamin is water soluble and has no known toxicity; and (b) as an indispensable vitamin for sustaining life, it is unlikely that a mutational arrest of cobalamin uptake could occur with concomitant failure of vitamin-mediated therapy.

Studies dating back almost 40 years showed high accumulation of radioactive ⁵⁷Co-vitamin B12 in transplanted tumors in mice using whole-body autoradiography. The authors state that “one practical consequence of this may be the possibility of using labeled vitamin B12 to locate tumors clinically” (1). Unfortunately, the long half-life of ⁵⁷Co with 272 days limits the maximal dose in human investigations to 37 kBq (1 µCi) per study, which made external imaging impossible. Furthermore, high accumulation of radioactivity in the liver, the pancreas, and especially in the kidneys was also observed. Nevertheless, recently, cobalamin derivatives labeled with short-lived radionuclides have emerged. Occult tumors in mice, pigs, dogs, and humans could be visualized via ¹¹¹In- and ^{99m}Tc-radiolabeled diethylenetriaminepentaacetate cobalamin analogues (DTPA-Cbl; ref. 2). But again, the greatest focal uptake of these cobalamin derivatives occurred in the liver, kidney, and spleen followed by the nasal cavity and salivary and lachrymal glands.

We undertook a new effort to improve targeting of radiolabeled cobalamin with different analogues (derivatization at the ribose part, the cyanide ligand, or use of a more stable chelating system; refs. 3–7) but failed initially to decrease high kidney and liver uptake due to the intrinsic properties of cobalamin to be stored in these organs (8). Therefore, another approach was required to interfere with the storage function of these organs.

Mammals must acquire cobalamin from food where it is present in extremely minute quantities. For these reasons, its reabsorption is entirely based on the association of cobalamin with soluble transport proteins in circulation, namely transcobalamin I (TCI; synonyms: haptocorrin and vitamin B12 R binder), intrinsic factor (IF), and transcobalamin II (TCII) and the corresponding cellular receptors. Cobalamin homeostasis involves adequate transport through the stomach of cobalamin bound to TCI, subsequent enzymatic release of cobalamin in the gut, where cobalamin is immediately rebound by IF and transported as IF-Cbl-complex across the intestinal epithelium to the blood stream (9). Here, cobalamin is bound by apo-TCII, the principal B12-binding protein (10) and becomes holo-TCII. Cellular uptake of holo-TCII is mediated via the endocytotic receptor TCII-R, inducing ubiquitous internalization of cobalamin (11). The highest level of TCII-R expression in human tissue is observed in the kidney, whereas lower levels of expression are found in the intestine, liver, and placenta. Another receptor, megalin, is strongly expressed in the kidneys where it mediates reabsorption and storage of holo-TCII (12–14). It has been suggested that this kidney tubular cobalamin reabsorption is similar in efficacy to the intestinal uptake (15). Megalin is also exclusively expressed in all glands and absorptive epithelia (16) to absorb holo-TCII.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

R. Waibel and H. Treichler contributed equally to this work.

Requests for reprints: Robert Waibel, Center for Radiopharmaceutical Science, Paul Scherrer Institute, CH-5232 Villigen PSI, Switzerland. Phone: 56-310-2826; Fax: 56-310-2849; E-mail: robert.waibel@psi.ch.

©2008 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-07-6771

For clinical application of radiopharmaceuticals, it is known that high kidney uptake of radiolabeled compounds may lead to radiation toxicity. Because the radiation dose to the kidney is often the dose-limiting factor for application of radiopharmaceuticals, we set out to investigate the possibility of preventing high organ uptake by disrupting the binding of cobalamin to its transport protein TCII and, therefore, inhibit uptake mediated by the receptors TCII-R and megalin. By abolishing binding of cobalamin to TCII (17), a decreased nontargeted organ uptake can be expected. Furthermore by interfering with the binding of cobalamin with circulating TCII, radioactive cobalamin as a small molecule should clear much faster from the blood than the protein-bound cobalamin, resulting in lower systemic toxicity.

Our new cobalamin derivatives still have vitamin function and undisturbed interaction with IF and TCI. We hypothesized that an alternative way of cobalamin binding to tumors might be through TCI fixation because it is known from the literature that some tumors express high levels of cytoplasmic and membrane-bound TCI (18). Therefore, we screened clinical samples of human cancer tissues (tissue microarrays; TMA) with a monoclonal antibody against TCI to define high TCI-expressing tumors. These tumors will be preferentially selected in a planned phase I clinical study with radiolabeled cobalamin derivative.

Materials and Methods

Synthesis of cobalamin monocarboxylic acids derivatives. Coupling of functionalized PAMA derivatives to cobalamin requires a reactive group on vitamin B12. Controlled acid hydrolysis of cobalamin in 0.1 mol/L HCl yields a mixture of products, with the *b*-, *d*-, and *e*-acids making up the largest portion. These can be obtained in pure form via an elaborate purification method, involving ion-exchange chromatography and preparative high performance liquid chromatography (HPLC; ref. 17). Starting with 1.9 g cobalamin (1.39 mmol), Cbl-*b*-acid was isolated in a yield of 280.6 mg (14.9%), Cbl-*d*-acid in a yield of 131.5 mg (7.0%), and Cbl-*e*-acid in a yield of 94.26 mg (5.0%). The bifunctional, tridentate metal chelating system (*N*-3-aminopropyl-*N'*-pyridin-2-ylmethyl-amino)acetic acid ethyl ester was prepared as described for the pentyl analogue by Schibli and coworkers (3). The compound is prone to cyclize under basic conditions. Therefore, the *t*-butoxycarbonyl (Boc)-protected intermediate was stored, and Boc was removed just before further functionalization by stirring in diluted aqueous HCl. The ethyl, butyl, pentyl, and hexyl derivatives were prepared in an analogous way. The Boc-deprotected PAMA ligands were coupled to Cbl-*b*-acid either by using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride as coupling reagent in water at a pH of 5.5 or by using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) as coupling reagent in a mixture of *N,N*-dimethylformamide, DMSO, and triethylamine. The products were isolated by preparative HPLC in yields of 33% to 63%. Analytic data and the X-ray structures for Tc(3)-Cbl and its nonradioactive Re-complex, Re(3)-Cbl, can be found in a recently published paper(19).

Derivatization by DTPA. The DTPA-Cbl analogue, cyanocobalamin-*b*-(4-aminobutyl)-DTPA was synthesized as described elsewhere (2).

Labeling procedures. [Na]^{99m}TcO₄ was eluted from a ⁹⁹Mo/^{99m}Tc generator (Mallinckrodt-Tyco) with a 0.9% saline solution. Precursor [^{99m}Tc(CO)₃(OH₂)₃]⁺ was prepared using the Isolink kit (Mallinckrodt-Tyco; ref. 20, 21). Chelating of the precursor to Cbl-*b*-(ethyl-hexyl)-PAMA-OEt was performed in a 10-mL glass vial with rubber stopper that was flushed with N₂. Twenty microliters of a solution of cobalamin derivative (0.001 mol/L in water), 20 μL or 200 μL MES buffer (pH 6.2; final concentration, 0.1 mol/L), and 160 μL or 1,780 μL of a [^{99m}Tc(OH₂)₃(CO)₃]⁺ solution were added, and the reaction mixture was kept at 75°C for 1 to 2 h. The final cobalamin concentration was 10⁻⁴ to 10⁻⁵ mol/L. HPLC analyses with γ-detection were performed to verify full conversion of the ^{99m}Tc species. Under these

conditions, ester-protecting groups in the chelators were cleaved to give the carboxylate complexes.

For *in vivo* biodistribution studies and for *in vitro* binding studies to the transport vectors, high specific activity was required. Therefore, radioactive-labeled cobalamin derivative was separated from unlabeled cobalamin on an analytic HPLC system. Separation conditions were as follows: XTerra RP8 column, gradient; acetate buffer, 10% ethanol (0 min), 25% ethanol (15 min), and 25% ethanol (25 min) for the *b*-system. ¹¹¹In labeling of Cbl-*b*-DTPA was prepared as described (2). ⁵⁷Co-cyanocobalamin (10.5 μCi/50 ng/mL) was purchased from MP Biomedicals.

Source of transport proteins TCI, TCII, and IF; preparation of TCI (haptocorrin). As a source of TCI, saliva of vegetarian human subjects was used. Saliva was centrifuged at 20,000 rpm 20 min at 4°C, mixed 1:1 with PBS and sterile filtered. The binding capacity of TCI was usually 400 ng ⁵⁷Co-cyanocobalamin/mL.

Preparation of apo-TCII. This recombinant human protein was obtained commercially (Tynes Scientific Proteins). Unfortunately, this protein was prone to aggregate, and therefore, for practical reasons, a supply of apo-TCII was also purified from rabbit whole blood (22). TCII was purified by affinity chromatography on a cyanocobalamin-agarose matrix (Sigma). Two hundred milliliters of twice centrifuged fresh whole blood (first time, 5,000 rpm for 15 min; second time, 20,000 rpm for 30 min at 4°C) was applied to the affinity column, and the column was washed sequentially. Bound TCII was eluted with 20 mL 4 mol/L guanidine HCl in 50 mmol/L Tris buffer (pH 8.0) and, in a second step, with 7.5 mol/L guanidine HCl in 50 mmol/L Tris buffer (pH 8.0). Most of the bound TCII eluted already with 4 mol/L guanidine HCl, and only this fraction was used. Probes were dialyzed extensively against H₂O for 2 d at 4°C. Typical yields are 5 to 30 nmol/L that translates into 7.5 to 10 μg of TCII (molecular weight, 45 kDa) per rabbit. The binding capacities of the dialyzed samples were assessed with ⁵⁷Co-cyanocobalamin and were around 50 ng/mL probe.

IF from porcine gastric mucosa, with a binding capacity of 1 ng of ⁵⁷Co-cyanocobalamin per unit of IF, was purchased from Sigma.

Interaction of cobalamin derivatives with transport proteins TCI, TCII, and IF. The interaction of radiolabeled (⁵⁷Co, ^{99m}Tc, and ¹¹¹In) cobalamin derivatives with the transport proteins TCI, TCII, and IF was measured by a gel-shift assay. Radiolabeled cobalamin (0.05–1 ng) was allowed to react with an excess of transport proteins for 15 min at room temperature. This mixture was applied to a gel-filtration column (Superdex75; Amersham Biosciences) with the running buffer PBS and 0.1% Tween 20. Biologically active cobalamin, which binds to transport proteins, shifts from a molecular weight of ~1.4 kDa to 40 to 70 kDa, depending on the transport protein. Titration of the binding capacity of the transport proteins was done with ⁵⁷Co-cyanocobalamin.

Cell cultures. For the biodistribution experiments in mice, the following cells were cultivated: melanoma cells B16F10 [CRL-6475; American Type Culture Collection (ATCC)], MEL-CLS-1 (melanoblastoma), MEL-CLS-2 (melanotic), MEL-CLS-3 (amelanotic), MEL-JUSO (metastatic; all from Cell-Lines-Service),⁸ and M1 (melanoma; University Hospital Zurich); bladder carcinoma UM-UC-3 (CRL-1749; ATCC) and HT1376 (CRL-1472; ATCC); kidney carcinoma Caki-2 (HTB-47; ATCC) and Fohn (University Hospital Zurich); small cell lung carcinoma SW2 (Dana Faber Cancer Institute); pancreas carcinoma MIA PaCa-2 (CRL-1420; ATCC); colon carcinoma LS 147T (CRL-188; ATCC); and prostate carcinoma LNCaP (CRL-1740; ATCC), neuroblastoma SK-N-AS (CRL-2137; ATCC), and mouse sarcoma S-180 II (CCL-8; ATCC).

Biodistribution of radiolabeled cyanocobalamin derivatives in mice. Four- to five-week-old female, athymic nude mice (CD1-Foxn1/nu) and BALB/c mice were purchased from Charles River Laboratories. Mice were housed under conditions of controlled temperature (26°C), humidity (68%), and daily light cycle (12 h light/12 h dark). Mice experiments were approved by the Animal Ethics Committee of the Kanton of Aargau (Nr.75528). To reduce interference of high Vitamin B12 levels in mice, the animals were fed with a Vitamin B12-deficient rodent diet (Harlan Teklad) for 5 wk.

⁸ <http://www.cell-lines-service.de>

Afterwards, they were inoculated s.c. with the tumor cell suspension (5×10^6 cells) into the subcutis of the axilla. Radiotracer distribution studies were performed 12 to 14 d after tumor cell inoculation. The radiotracers were administered via a lateral tail vein. The animals were sacrificed and dissected 1, 4, and 24 pi of the radiotracer. The selected tissues were removed, weighed, and counted for radioactivity to determine distribution of radioactivity within the test animal. The results were tabulated as percentage of the injected dose per gram (% ID/g) of weight tissue, using a reference sample. For biodistribution studies with ^{57}Co -cyanocobalamin, 7.4 kBq (0.2 μCi)/1 ng of the radiolabeled cyanocobalamin was mixed with normal saline and injected i.v. in tumor-bearing BALB/c mice (syngeneic mouse melanoma B16F10). After a specified time (5 min to 24 h), animals are sacrificed, and the organs were weighed and counted on a gamma counter. For biodistribution studies with $^{99\text{m}}\text{Tc}$ -labeled cyanocobalamin, 1.85 MBq (50 μCi)/(1–5 ng) of the radiolabeled cyanocobalamin was mixed with normal saline and used as before. For biodistribution with ^{111}In -labeled cyanocobalamin, 74 kBq (2 μCi)/5 ng of the radiolabeled cyanocobalamin was mixed with normal saline and used as before. To study the effect of Vitamin B12 deficient food, the biodistribution of labeled cyanocobalamin is compared in mice fed with normal food with the biodistribution in mice fed with vitamin B12-deficient food for a period of 5 wk.

Single-photon emission computed tomography imaging studies. Imaging experiments were performed with an X-SPECT system (Gamma Medica, Inc.) with a single head single-photon emission computed tomography (SPECT) device and a computed tomography (CT) device, 24 h pi of the radiotracer. The radiotracer [450–600 MBq (12–16 mCi)] was administered via a lateral tail vein. The mice were anesthetized with an isoflurane/oxygen mixture and positioned in the SPECT camera using the therefore intended animal bed. Depth of anesthesia was monitored by measuring respiratory frequency. Body temperature was controlled by a rectal probe and kept at 37°C by a thermo coupler and a heated air stream. In some cases, imaging studies were performed postmortem. SPECT data were acquired and reconstructed by the software LumaGEM (version 5.407; lum 10). CT data were acquired by the X-Ray CT system Gamma Medica and reconstructed by the software Cobra (version 4.5.1). Fusion of SPECT and CT data were performed by the software IDL Virtual Machine (version 6.0). Images were generated by Amira (version 3.1.1).

Microbial agar diffusion test. To test the ability of the cobalamin derivatives to sustain growth and therefore act as a vitamin, *Lactobacillus delbruekii* var. *lactis* (ATCC 7830) was used. Inoculation cultures of this B12-auxotrophic bacterial strain were washed thrice in sterile saline and added to a solution of vitamin B12 assay broth base (Merck) mixed with agar noble (Difco) and poured into Petri dishes. After solidifying, sterile filter discs (Sensi-Disc; Becton Dickinson) were placed on the surface of the medium. Solutions of Cbl derivatives and cyano-cobalamin as a control were applied on these discs. Quantitative measurements were carried out to determine the proportion (in percent) by which the respective derivatives were able to promote growth and therefore fulfill the catalytic action of cobalamin.

Immunohistochemistry. For analysis of TCI expression on tumors, immunostaining with anti-TCI polyclonal antibody [chicken anti-TCN1 (ab14273); Abcam Limited] was performed on a TMA. The construction of the TMA has been reported elsewhere (23). The antibody was diluted 1:50 and incubated overnight at 4°C. Deparaffined sections of the TMA were manually stained after heat-induced epitope retrieval [10 min; 110°C; citrate buffer (pH 6.0)] using a standard multilink detection kit (Medi-Stain HRP DAB; mediateAG), including endogenous peroxidase block, block of nonspecific binding, horseradish peroxidase, and diaminobenzidine as chromogen. The TCI expression on the TMA was semiquantitatively assessed by one observer (NGS) and assistance was received for ambiguous cases (HM). The staining pattern was confirmed on every individual spot. In a first step, staining intensity of positive tumor cells was recorded as follows: weak (1+), moderate (2+), or strong (3+). In a second step, the percentage of positive tumor cells was calculated and a three-tiered score (negative, weak, and strong staining) was generated for statistical analysis. All tumors without staining or staining in up to 10% of tumor cells were regarded as negative. Tumors with 1+ staining intensity in >10% or 2+ staining intensity in 11% to 50% were considered weakly positive. Spots with

2+ intensity in >50% of the tumor cells or 3+ intensity in >10% of tumor cells were considered strongly positive. The relatively high cutoff value of 10% was set to rule out false positive results. Cell nuclei were stained by hematoxylin.

Results

Interaction of radioactive cobalamin derivatives with transport proteins. The pathway of cobalamin uptake involves a series of transport proteins. The main transport proteins are TCI from saliva, IF in the gut, and the primary serum transport protein TCII. A series of cobalamin derivatives with increasing linker chain length between the cobalamin unit and the radiometallic unit ranging from ethyl to hexyl [Tc(2)-Cbl to Tc(6)-Cbl] were synthesized (Fig. 1) and tested for binding to these three transport proteins. All cobalamin derivatives were built from cyanocobalamin-*b*-acid, derived by mild acid hydrolysis of the acid amide side chain of cyanocobalamin. All conjugates carry the monoanionic PAMA-ligand ([pyridine-2-ylmethyl-amino]-acetic acid; ref. 3), with an NNO-donor set that can be quantitatively labeled with [$^{99\text{m}}\text{Tc}(\text{OH}_2)_3(\text{CO})_3$] $^+$ (yields >98%; refs. 24, 25). For comparison, the ^{111}In -DTPA-Cbl analogue, ^{57}Co -Cbl, Tc-Cbl (26), and Tc-PAMA were also included in these studies and tested for transport protein binding.

The interaction of the cobalamin derivatives with the transport proteins was measured by a gel-shift assay (Fig. 2). Radiolabeled cobalamin was allowed to react with an excess of transport proteins. This mixture was applied on a gel-filtration column to separate free activity from protein-bound activity. Biologically active cobalamin bound to transport proteins exhibit a molecular weight shift from ~1.5 kDa to 40 to 60 kDa, depending on the transport protein. As seen in Table 1, all cobalamin derivatives bound to TCI and IF. Short chain length Tc(2)-Cbl, Tc(3)-Cbl, and Tc(4)-Cbl lost their binding to TCII. By increasing the linker above

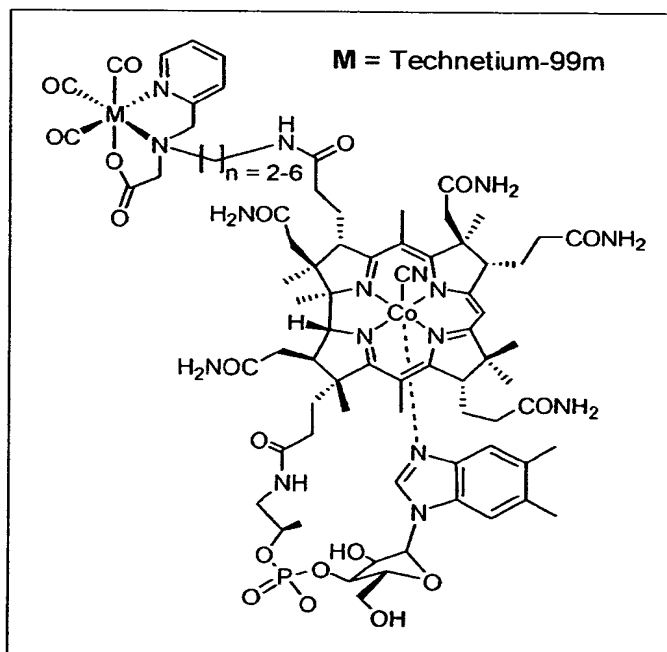


Figure 1. General chemical structure of the organometallic $^{99\text{m}}\text{Tc}$ -PAMA-Cbl radiotracers Tc(2)-Cbl to Tc(6)-Cbl.

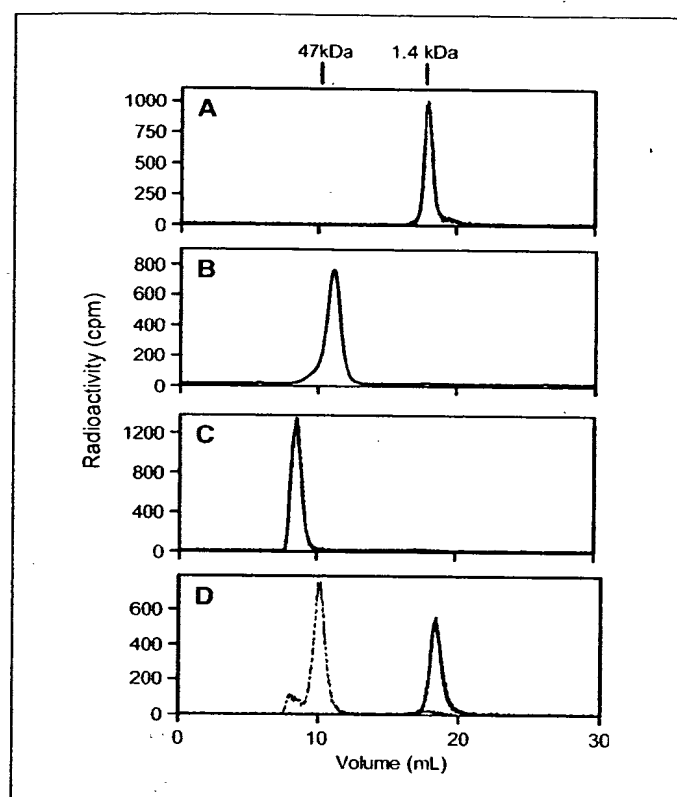


Figure 2. Gel-shift assay on Superdex75 gelfiltration column with radiolabeled Tc(4)-Cbl associated with or without their transport proteins. A, Tc(4)-Cbl only (1.4 kDa), Tc(4)-Cbl mixed with transport protein TCI (B; 47 kDa), Tc(4)-Cbl mixed with transport protein IF (C; 60 kDa), and Tc(4)-Cbl mixed with TCII (D). The elution peaks of radioactive cobalamin shifted with the binding to the transport proteins to higher molecular weight. Tc(6)-Cbl (dotted curve) was used as a positive control (TCII binder).

four, the derivatives regained the binding ability to TCII. ^{111}In -Cbl and ^{57}Co -Cbl interacted with all three transport proteins.

In vivo distribution of cobalamin derivatives. Our set of new cobalamin analogues was compared with the "native" ^{57}Co -Cbl and the clinically tested ^{111}In -DTPA-Cbl for tissue distribution and tumor uptake in mice carrying syngeneic B16F10 melanoma tumors. Tumor uptake of our best derivative Tc(4)-Cbl was not dependent on functional TCII binding and reached even higher values of percent injected dose per gram tissue than TCII binders

such as Tc(6)-Cbl, ^{111}In -Cbl, or ^{57}Co -Cbl (Supplementary Table S1; Fig. 3). When comparing Tc(4)-Cbl (TCII nonbinder) with Tc(6)-Cbl (TCII binder), all tissues showed statistically significant lower uptake of radioactivity, except for the tumor. Tumor to blood ratios of ~90:1 was achieved with the derivative Tc(4)-Cbl compared with ratios well below 10:1 for TCII binders. All TCII nonbinder derivatives displayed a fast blood clearance, with a half-life T_{α} of 2.2 min and T_{β} of 36 min for Tc(4)-Cbl. Because Tc(4)-Cbl still binds efficiently to TCI, we investigated the possibility that tumor uptake is mediated by TCI. We tested if another TCI binder, namely cobinamide, could interfere with the binding of Tc(4)-Cbl to melanoma tumors. Competition experiments with cobinamide completely blocked the uptake of labeled Tc(4)-Cbl with equal efficiency as free cobalamin (Supplementary Table S1).

A limited amount of other xenografted tumors were also tested in nu/nu mice. We scored a tumor as positive when >2% injected dose per gram of tissue was observed. In negative xenografts, <1% injected dose per gram of tissue accumulated at the tumor site. All six melanomas tested were positive; two bladder carcinomas, one pancreas carcinoma, one small cell lung carcinoma, and one of two renal carcinomas (clear cell) were positive, whereas colon (one), prostate (one), and neuroblastoma (one) were negative. Mouse sarcoma was also negative, although this tumor gave a positive enrichment with the ^{111}In -DTPA derivative.

One of the melanoma xenografts (CLS-1) showed a very high tumor uptake (up to 13% injected dose per gram of tissue) after 24 hours. In the same xenograft, high circulating activity was seen after 24 hours (up to 14%). In the literature, very high serum TCI levels have been found in patients with metastatic cancer. When analyzing serum of our mouse xenograft on a gel filtration sizing column, the radioactivity was associated with a molecular mass of 120 to 140 kDa, whereas in the mouse model with B16F10 tumors, only free cobalamin with a molecular mass of 1.5 kDa could be detected in serum.

TCI expression by immunohistochemical analysis of human tissues microarray. A plausible mechanism of cobalamin uptake could be the consequence of tumors expressing *de novo* binding proteins such as TCI. High expression of TCI on the tumor would help to satisfy the increased demand for cobalamin. Therefore, a multitissue TMA was used to analyze TCI expression in different tumor types and normal tissues. The TMA comprised a total of 184 tissue cylinders. One hundred thirty three tumor spots and 37 normal tissue spots were interpretable for TCI staining. TCI expression in positive tissues was detected in the cytoplasm of some normal epithelial cells and various tumor cells. Strong TCI positivity was found in seminoma of the testis, breast cancer

Table 1. Binding interaction of cobalamin analogues with transport proteins

	Tc(2)Cbl	Tc(3)Cbl	Tc(4)Cbl	Tc(5)Cbl	Tc(6)Cbl	^{111}In Cbl	^{57}Co Cbl	TcCbi	TcPAMA
TCI	+	+	+	+	+	+	+	+	-
IF	+	+	+	+	+	+	+	-	-
TCII	-	-	-	+	+	+	+	-	-

NOTE: Binding interaction of ^{99m}Tc -labeled Cbl analogues with increasing linker length [Tc(2)Cbl-Tc(6)Cbl] with TCI, IF, and TCII. ^{111}In -DTPA-Cbl (^{111}In Cbl), ^{57}Co -Cobalamin (^{57}Co Cbl), and ^{99m}Tc -cobinamide (TcCbi) were also tested. The ^{99m}Tc -PAMA complex lacking the Cbl moiety was used as a negative control.

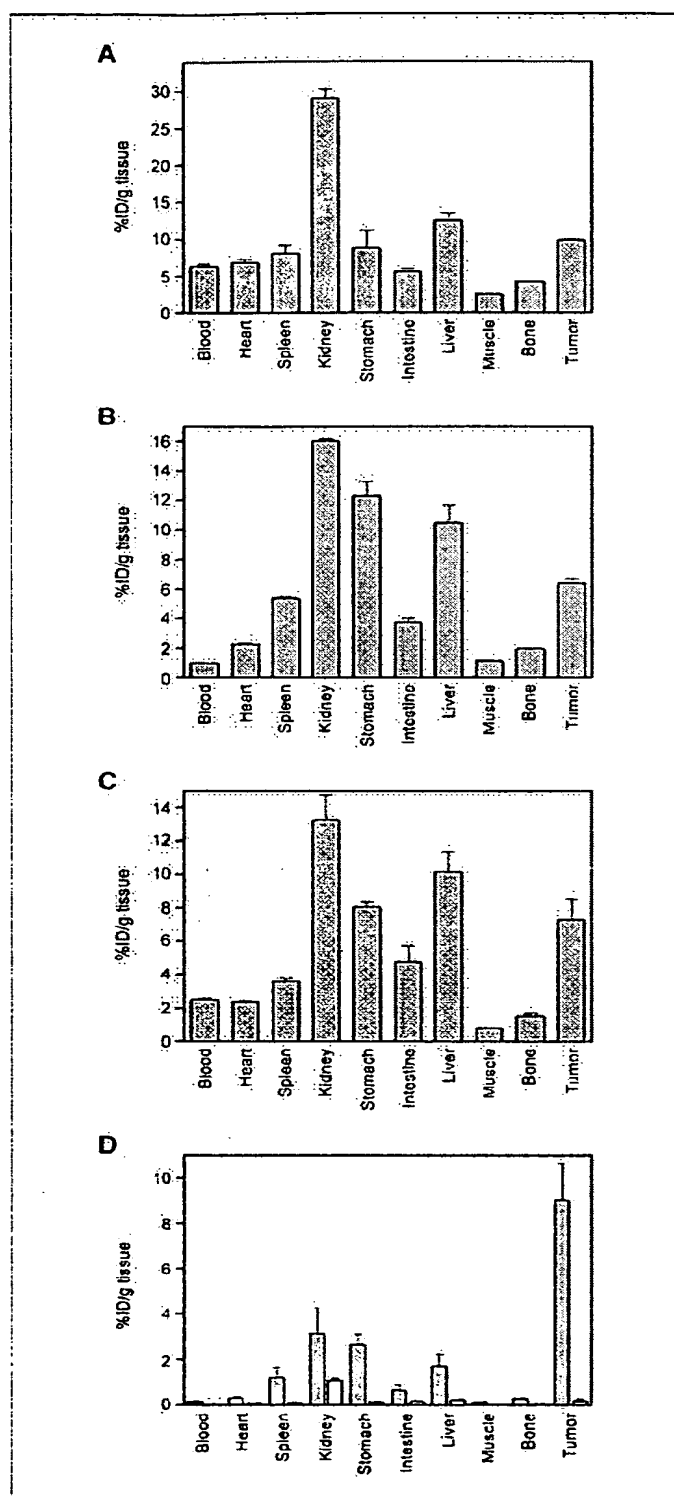


Figure 3. Organ distribution 24 h after i.v. injection of ^{57}Co -Cbl (A), of ^{111}In -DTPA-Cbl (B) and $^{99\text{m}}\text{Tc}$ -tricarbonyl labeled Tc(6)-Cbl (C), and of Tc(4)-Cbl (D) in mice carrying syngeneic B16F10 melanoma tumors. The doses of injected cobalamin were 1 to 20 ng per mouse. As a proof of specificity of the tracer uptake, 20 ng of radioactive Tc(4)-Cbl was mixed with 500 ng nonlabeled Tc(4)-Cbl or cobinamide to compete for organ enrichment [punctuated bars on the right hand of the main gray bars (D)].

(lobular, ductal, and mucinous), lung cancer (adenocarcinoma and squamous cell carcinoma), sarcoma (soft tissue sarcoma and myxofibrosarcoma), ovarian cancer (serous papillary, clear cell, and transitional cell), skin cancer (squamous cell), brain tumor (glioblastoma multiforme), thyroid cancer (anaplastic carcinoma), uterine cancer (endometroid adenocarcinoma and serous papillary carcinoma), malignant melanoma, kidney cancer (clear cell and papillary), prostate adenocarcinoma, bladder cancer, and lymphoma (mantle cell and diffuse large B cell). Strong TCI positivity of benign tissue was exclusively found in the liver. Weakly positive staining was found in testicular cancer (teratoma), hepatocellular adenocarcinoma, soft tissue sarcoma (synovial sarcoma, leiomyosarcoma), colon adenocarcinoma, thyroid cancer (papillary and follicular), and lymphoma (marginal zone). Normal tissue with weak TCI expression was renal tissue and the palatine tonsil. There was no TCI expression in pancreatic cancer (ductal adenocarcinoma, anaplastic carcinoma, and neuroendocrine cancer) and small cell lung cancer. Placenta and normal pancreas revealed also no TCI expression. The findings are exemplified in Fig. 4 and Supplementary Table S2.

SPECT/CT imaging studies. Combined SPECT/CT studies were performed with tumor-bearing mice with a dedicated small animal SPECT/CT scanner. Scan time varied between 30 and 60 minutes. Whole body SPECT/CT of a mouse bearing a melanoma tumor at the neck was performed with Tc(4)-Cbl (Fig. 5A) 24 hours after i.v. injection and compared with a scan of a mouse with TCII-active Tc(6)-Cbl (Fig. 5B). These pictures clearly confirm the different tissue distribution between a TCII nonbinder and binder derivative, showing only radioactivity in the tumor, kidney, and intestine for the nonbinder Tc(4)-Cbl, whereas with the binder Tc(6)-Cbl, the radioactivity is systemically distributed. The SPECT/CT scans with a high resolution, low sensitivity pinhole collimator of the head of mice injected with Tc(4)-Cbl and Tc(6)-Cbl are shown in Fig. 5C and D and confirms the striking difference in accumulation of the two molecules. Tc(4)-Cbl was only enriched at the tumor site, whereas Tc(6)-Cbl also accumulated at sites with high megalin content such as thyroid, lachrymal glands, and salivary glands.

Vitamin function of Tc(3)-Cbl and Tc(4)-Cbl. We selected a vital vitamin as radiopharmaceutical, because its pivotal uptake should prevent any kind of mutations leading to a diminished import of this vitamin. To test the derivatives for vitamin function, we used a microbial growth assay. The internationally recommended tester strain for vitamin B₁₂, *Lactobacillus delbruekii* (ATCC 7830; ref. 27) was applied to study the derivatives for their activity as a vitamin. Supply of normal cobalamin or Tc(3)-Cbl and Tc(4)-Cbl to this B₁₂-auxotrophic bacterial strain in a vitamin B₁₂-free assay medium resulted in a growth response, as measured by a quantitative solid diffusion plate assay. This assay showed a functional catalytic activity of 20% for Tc(3)-Cbl and 10% for Tc(4)-Cbl when compared with normal cobalamin. These derivatives can still act as key growth factors.

Discussion

Vitamin B₁₂-mediated targeting as a mechanism to increase drug uptake by tumors was always thought to be dependent on undisturbed interaction of cobalamin with the main transport proteins IF, TCI, and TCII. Earlier reported derivatives were selected on the assumption that high uptake in tumors can only be achieved through binding to TCII (2, 28–31).

Our previous efforts to improve cobalamin targeting of radio-labeled cobalamin with new analogues (derivatization at the ribose part or the cyanide ligand, or using a more stable chelating system) were centered on the same effort not to impair TCII recognition (5). Although TCII interaction was maintained, which can be explained by the overall structure of the recently crystallized holo-TCII (32), our data did not show an improvement in differentiation between normal tissue and tumor compared with already published results (2, 33). All these derivatives showed disturbing high renal accumulation.

Therefore, we completely changed the concept of tumor targeting by TCII receptor-mediated endocytosis and searched instead for derivatives that still show high tumor uptake but do not accumulate in the kidney. Megalin is responsible for Cbl-TCII endocytosis, and interrupting the binding of cobalamin to TCII should therefore also interrupt Cbl-TCII uptake. In the literature, cobalamin-biotin conjugates that vary in the position of cobalamin coupling have been described. Biotin derivatives at the *b*-side chain showed little interaction with TCII and were thought to be used for applications where decreased binding with transport proteins is desired (17). Thus, we synthesized and tested a set of cobalamin derivatized at the *b*-side chain with increasing chain length. Data published showed that increasing the spacer length beyond a certain length resulted in regained binding of derivatives to the transport proteins (34). Our own data showed no binding to TCII for the three derivatives with short chain length Tc(2)-Cbl, Tc(3)-

Cbl, and Tc(4)-Cbl but still maintaining tumor uptake. These three derivatives had strongly diminished binding to TCII, yet still were capable of interaction with TCI and IF and showed function as a vitamin. Increasing the chain length beyond butyl restored the interaction to TCII (Supplementary Table S1; Table 1) for Tc(5)-Cbl and Tc(6)-Cbl. Tc(4)-Cbl with tumor to blood ratio of 90 and tumor to kidney ratio of 2.6 was chosen as the best derivative. These data for Tc(4)-Cbl exceeds distinctly those of the clinically tested ^{111}In -DTPA-Cbl derivative with tumor to blood ratio of 6 and tumor to kidney ratio of only 0.5 (Fig. 3).

Experiments with Tc(4)-Cbl derivative with no TCII binding showed that malignant and fast growing tumors such as melanoma are no longer dependent on the transport protein TCII to mediate high uptake of cobalamin. Because Tc(4)-Cbl still binds efficiently to TCI, we investigated the possibility that tumor uptake is mediated by TCI. A plausible mechanism of Cbl-uptake could be the consequence of tumors expressing *de novo* binding proteins such as TCI. High expression of TCI on the tumor would help to satisfy the increased demand for cobalamin. High levels of the cobalamin-binding protein TCI have been described in malignancies such as cancer of the breast, colon, pancreas, lung, liver, kidney, salivary gland, stomach, and endometrial adenocarcinoma (35–40). It has been suggested that tumors are capable of producing TCI (41, 42) themselves. Immunohistochemically, TCI has recently been localized to the membrane and cytoplasm of tumor cells (43). Therefore, we extended the database of TCI

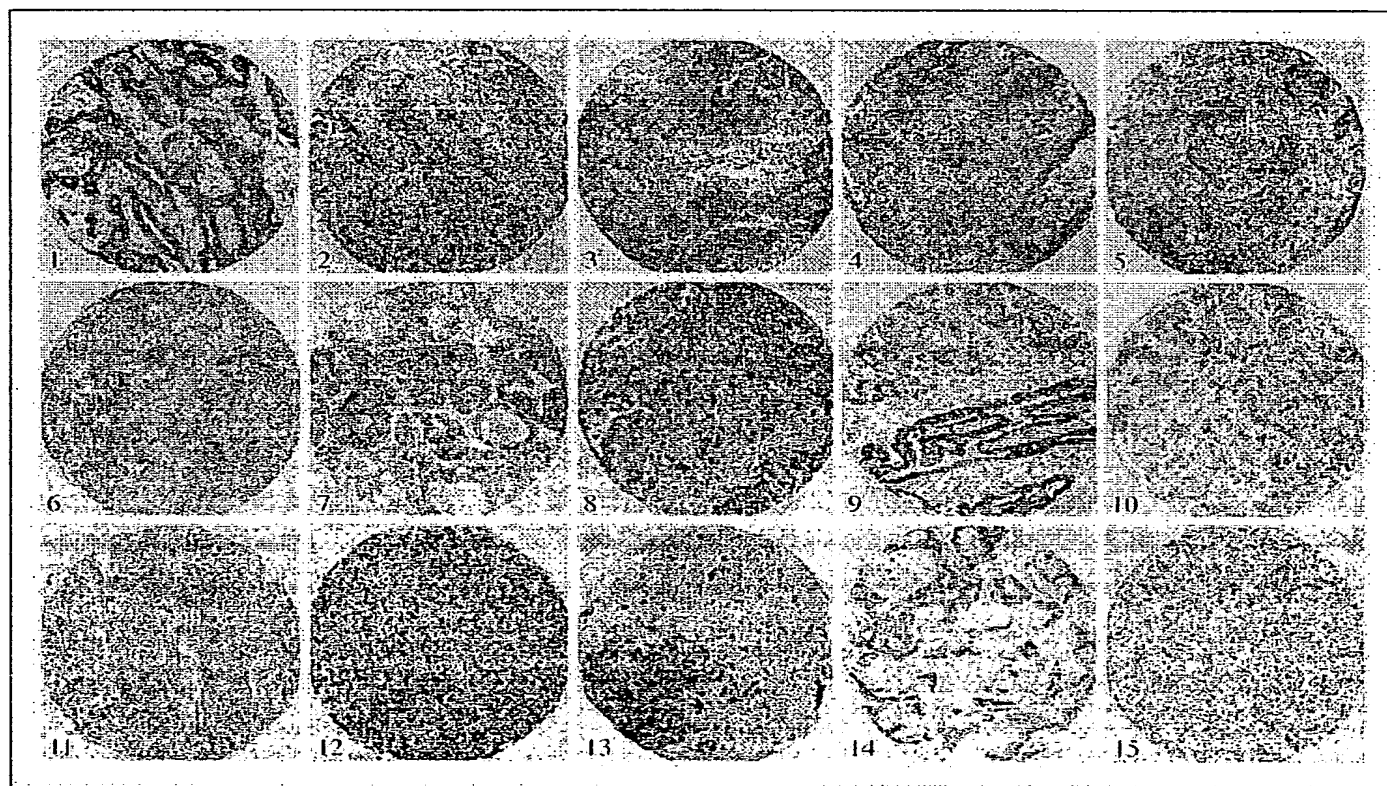


Figure 4. TCI expression by immunohistochemical analysis. Multitumor human TMA. TCI expression of tissue was shown by immunohistochemistry with a polyclonal anti-TCI antibody (brown). The cell nuclei were stained with hematoxylin (blue). Tumor tissues are as follows: lung adenocarcinoma (1), seminoma of the testis (2), malignant melanoma (3), skin squamous cell cancer (4), bladder urothelial cancer (5), soft tissue sarcoma (6), prostate adenocarcinoma (7), serous papillary adenocarcinoma of the uterus (8), clear cell adenocarcinoma of the ovary (9), hepatocellular adenocarcinoma (10), diffuse large B-cell lymphoma (11), clear cell kidney cancer (12), glioblastoma multiforme (13), lobular breast cancer (14), and anaplastic thyroid cancer (15).

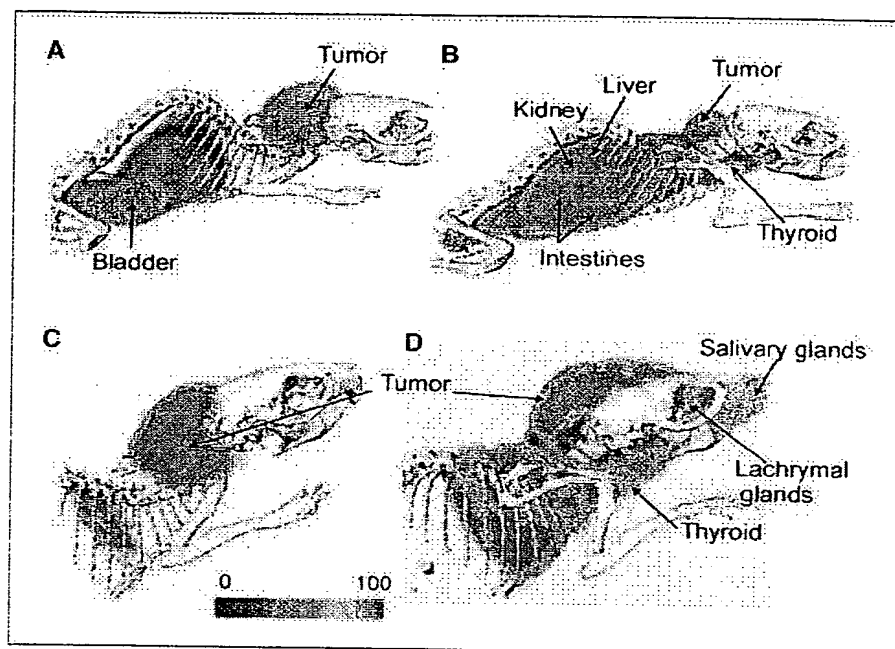


Figure 5. Whole body SPECT/CT scans 24 h after i.v. injection. **A**, three-dimensional picture of a mouse with a B16F10 tumor at the neck injected with Tc(4)-Cbl (TCII nonbinder, low resolution, high sensitivity). **B**, three-dimensional picture of a mouse with a B16F10 tumor injected with Tc(6)-Cbl (TCII binder). **C**, SPECT/CT (high resolution, low sensitivity, pinhole collimator) scan of the head 24 h after i.v. injection with Tc(4)-Cbl. **D**, SPECT/CT (high resolution, low sensitivity, pinhole collimator) scans of the head 24 h after i.v. injection with Tc(6)-Cbl. The color scale is normalized to the highest activity.

expression in tumor and benign tissues by screening TMAs with an antibody against TCI. When analyzing 170 different tumors and normal tissues, we confirmed and expanded the previous data on high TCI expression in a multitude of tumors (Fig. 4; Supplementary Table S2).

The nature of the uptake mechanism was further investigated by competing the binding of Tc(4)-Cbl to melanoma tumors with the cobalamin precursor cobinamide. Cobinamide also binds strongly to TCI with no binding to IF or TCII. Competition experiments with cobinamide injected 4 hours before Tc(4)-Cbl application completely blocked the binding at the tumor site of Tc(4)-Cbl with equal efficiency as free cobalamin. This experiment suggests that binding to the tumor site is indeed TCI mediated.

Abolishing binding of cobalamin to the transport protein TCII also completely destroyed binding of Cbl-TCII to megalin. Megalin is not able to bind free cobalamin but only cobalamin complexed with TCII. This lack of binding has the consequence that tissues with high megalin expression, especially kidney, glands, and absorptive epithelia show a much lower or no uptake at all of Cbl-derivatives Tc(2)-(4). As shown in Fig. 5, there is a striking difference in tissue distribution between a TCII nonbinder Tc(4)-Cbl (Fig. 5A) and a TCII binder Tc(6)-Cbl molecule (Fig. 5B). The eliminated megalin interaction is responsible for the lack of

binding to thyroid glands (44), eyes, and salivary glands as seen in the high resolution SPECT scans (Fig. 5C and D).

In conclusion, we have synthesized a set of cobalamin analogues with abolished interaction with TCII. These analogues still showed vitamin function. Blocking TCII interaction resulted in decreased nontargeted binding to organs predestined to have high cobalamin uptake. Binding to TCI and IF remained unchanged. This pattern of interaction is unique and not expected from experimental (45–47) and theoretical data (32, 48). Exploiting an alternative way of cobalamin uptake, most likely TCI-mediated, high tumor accumulation of Tc(4)-Cbl could be observed in a subset of malignancies. The low systemic distribution of radioactivity and the high tumor to blood ratio opens the possibility of a promising clinical application for cobalamin. Clinical trials to prove this concept will be initiated by the Paul Scherrer Institute in collaboration with the University Hospital Zurich.

Acknowledgments

Received 12/20/2007; revised 2/7/2008; accepted 2/8/2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Jürgen Grünberg and Christine De Pasquale for excellent technical assistance and Alexander Hohn for his support with the SPECT/CT studies.

References

- Ullberg S, Flodh H. Accumulation of labeled vitamin B12 in some transplanted tumours. *Int J Cancer* 1968;3: 694–9.
- Collins DA, Hogenkamp HPC, O'Connor MK, et al. Biodistribution of radiolabeled adenosylcobalamin in patients diagnosed with various malignancies. *Mayo Clin Proc* 2000;75:568–80.
- Stüchelberger A, Waibel R, Dumas C, Schubiger PA, Schibli R. Versatile synthetic approach to new bifunctional chelating agents tailor made for labeling with the fac-[M(CO)₃]⁺ core (M = Tc, ^{99m}Tc, Re): synthesis, *in vitro*, and *in vivo* behavior of the model complex [M(APPA)(CO)₃] (APPA = [(5-amino-pentyl)-pyridin-2-yl-methyl-amino]-acetic acid). *Nuc Med Biol* 2003;30: 465–70.
- van Staveren DR, Benny PD, Waibel R, Kurz P, Pak JK, Alberto R. S-functionalized cysteine: powerful ligands for the labelling of bioactive molecules with triaquatrichloronitro-99m(1+) ([^{99m}Tc(OH₂)₃(CO)₃]⁺). *Helv Chim Acta* 2005;88:447–60.
- van Staveren DR, Waibel R, Mundwiler S, Schubiger PA, Alberto R. Conjugates of vitamin B12 with N-ε-function-
- alized histidine for labeling with [^{99m}Tc(OH₂)₃(CO)₃]⁺: synthesis and biodistribution studies in tumor bearing mice. *J Organomet Chem* 2004;689:4803–10.
- Kunze S, Zobi T, Kurz P, Springer B, Alberto R. Vitamin B12 as a ligand for technetium and rhenium complexes. *Angew Chem Int Ed* 2004;43:5025–9.
- van Staveren DR, Mundwiler S, Hoffmanns U, et al. Conjugation of novel histidine derivative to biomolecules and labelling with [^{99m}Tc(OH₂)₃(CO)₃]⁺. *Org Biomol Chem* 2004;2:2593–603.
- Birn H, Nexø E, Christensen EI, Nielson R. Diversity in rat tissue accumulation of vitamin B12 supports a

- distinct role for the kidney in vitamin B12 homeostasis. *Nephrol Dial Transplant* 2003;18:1095-100.
9. Chemistry and Biochemistry of B12. In: Banerjee R, editor. New York: John Wiley and Sons, Inc; 1999.
 10. Seetharam B, Bose S, Li NL. Cellular import of cobalamin (vitamin B12). *J Nutr* 1999;129:1761-4.
 11. Seetharam B. Receptor-mediated endocytosis of cobalamin (vitamin B12). *Annu Rev Nutr* 1999;19:173-5.
 12. Birn H, Willnow TE, Nielsen R, et al. Megalin is essential for renal proximal tubule reabsorption and accumulation of transcobalamin-B12. *Am J Physiol Renal Physiol* 2002;282:F408-16.
 13. Moestrup SK. New insights into carrier binding and epithelial uptake of the erythropoietic nutrients cobalamin and folate. *Curr Opin Hematol* 2006;13:119-23.
 14. Yammani RR, Seetharam S, Dahme NM, Seetharam B. Transcobalamin II receptor interacts with megalin in the renal apical brush border membrane. *J Membr Biol* 2003;193:57-66.
 15. Christensen EI, Birn H. Megalin and cubulin: synergistic endocytotic receptors in renal proximal tubule. *Am J Physiol Renal Physiol* 2001;280:F562-73.
 16. Christensen EI, Birn H. Megalin and cubulin: multifunctional endocytotic receptors. *Nature Rev* 2002;3:258-68.
 17. Pathare PM, Wilbur DS, Heusser S, Quadros EV, McLoughlin P, Morgan AC. Synthesis of cobalamin-biotin conjugates that vary in the position of cobalamin coupling. Evaluation of cobalamin derivative binding to transcobalamin II. *Bioconjugate Chem* 1996;7:217-32.
 18. Carmel R. Extreme elevation of serum transcobalamin I in patients with metastatic cancer. *N Engl J Med* 1975;292:282-4.
 19. Spingler B, Mundwiler S, Ruiz-Sanchez P, van Staveren D, Alberto R. Structures of *b*- and *d*-acid derivatives of vitamin B12 and their complexes with $[M(CO)_5]^+$ ($M = {}^{99m}\text{Tc-Re}$). *Eur J Inorg Chem* 2007;18:2641-7.
 20. Alberto R, Schibli R, Egli A, et al. Metal carbonyl syntheses XXII. Low pressure carbonylation of $[MOCl_4]^-$ and $[MO_4]^-$: the technetium(I) and rhenium(I) complexes $[NEt_4][MCl_5(CO)_5]$. *J Organomet Chem* 1995;493:119-27.
 21. Alberto R, Ortner K, Wheatley N, Schibli R, Schubiger PA. Synthesis and properties of boranocarbonate: a convenient *in situ* CO source for the aqueous preparation of $[{}^{99m}\text{Tc}(\text{OH}_2)_5(\text{CO})_5]^+$. *J Am Chem Soc* 2001;123:3135-6.
 22. Allen RH, Majerus PW. Isolation of vitamin B12-binding proteins using affinity chromatography. 3. Purification and properties of human plasma transcobalamin II. *J Biol Chem* 1972;247:7709-17.
 23. Kononen J, Buhendorf L, Kallioniemi A, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 1998;4:844-7.
 24. Alberto R, Schibli R, Egli A, et al. A novel organometallic aqua complex of technetium for the labeling of biomolecules: synthesis of $[{}^{99m}\text{Tc}(\text{OH}_2)_5(\text{CO})_5]^+$ from $[(\text{TCO}_4)]^-$ in aqueous solution and its reaction with a bifunctional ligand. *J A Chem Soc* 1998;120:7987-8.
 25. Waibel R, Alberto R, Willuda J, et al. Stable one-step technetium-99m labeling of His-tagged recombinant proteins with a novel $\text{Tc}(\text{I})$ -carbonyl complex. *Nat Biotechnol* 1999;17:897-901.
 26. Mundwiler S, Waibel R, Spingler B, Kunze S, Alberto R. Picolyamine-methylphosphonic acid esters as tridentate ligands for the labeling of alcohols with the fac- $[M(\text{CO})_3]^+$ core ($M = {}^{99m}\text{Tc-Re}$): synthesis and biodistribution of model compounds and of a ${}^{99m}\text{Tc}$ -labeled cobinamide. *Nuc Med Biol* 2005;32:473-84.
 27. Cuthbertson WF, Peger HF, Lloyd JT. The assay of vitamin-B12. Part III. Microbiological estimation with *Lactobacillus-lactis* Dorner by the plate method. *Analyst* 1951;76:133-41.
 28. McLean GR, Pathare PM, Wilbur DS, et al. Cobalamin analogues modulate the growth of leukemia cells *in vitro*. *Cancer Res* 1997;57:4015-22.
 29. Russell-Jones G, McTavish K, McEwan J, Rice J, Novotnik D. Vitamin-mediated targeting as a potential mechanism to increase drug uptake by tumours. *J Inorg Biochem* 2004;98:1625-33.
 30. Bagnato JD, Eilers AL, Horton RA, Grissom CB. Synthesis and characterization of a cobalamin-colchicine conjugate as a novel tumor-targeted cytotoxin. *J Organ Chem* 2004;69:8987-96.
 31. Bauer JA, Morrison BH, Grane RW, et al. Effects of interferon β on transcobalamin II-receptor expression and antitumor activity of nitrosylcobalamin. *J Nat Cancer Inst* 2003;94:1010-9.
 32. Wuerges J, Garau G, Geremia S, Fedosov SN, Petersen TE, Randaccio L. Structural basis for mammalian vitamin B12 transport by transcobalamin. *Proc Natl Acad Sci USA* 2006;103:4386-91.
 33. Yang JQ, Li Y, Lu J, Wang XB. Preparation and bio-distribution in mice of ${}^{99m}\text{Tc}$ -DTPA-b-cyanocobalamin. *J Radioanal Nucl Chem* 2005;265:467-72.
 34. Russell-Jones GJ. Vitamin B12 mediated oral delivery systems for granulocyte-colony stimulating factor and erythropoietin. *Bioconjugate Chem* 1995;6:459-65.
 35. Gimsing P, Hippe E. Increased concentration of transcobalamin I in a patient with metastatic carcinoma of the breast. *Scand J Haematol* 1978;21:243-9.
 36. Burger RL, Waxman S, Gilbert GS, Mehlmann CS, Allen RH. Isolation and characterization of a novel vitamin B12-binding protein associated with hepatocellular carcinoma. *J Clin Invest* 1975;56:1262-70.
 37. Ogawa K, Ogawa O, Koshiha M, et al. Immunohistochemical localization of vitamin B12 R-binder in salivary gland tumors. Implications for cell-differentiation. *Pathol Res Pract* 1990;86:751-8.
 38. Ogawa K, Kudo H, Kim YC, et al. Immunohistochemical distribution of vitamin B12 R-binder in renal cell carcinoma. *Virchows Arch A* 1987;412:23-6.
 39. Sheppard K, Bradbury DA, Davies JM, Ryrle DR. Cobalamin and folate binding proteins in human tumor tissue. *J Clin Pathol* 1984;37:1336-8.
 40. Ermens AAM, Vlasveld LT, Lindemans J. Significance of elevated cobalamin (vitamin B12) levels in blood. *Clin Biochem* 2003;36:585-90.
 41. Kudo H, Ohshio G, Ogawa K, et al. Distribution of vitamin B12 R-binder in carcinomas of the digestive tract. *J Clin Pathol* 1988;41:320-3.
 42. Wakatsuki Y, Inada M, Kudo H, et al. Immunological characterization and clinical implication of cobalamin binding protein in human gastric cancer. *Cancer Res* 1989;49:3122-8.
 43. Kim YC, Ogawa K, Wakatsuki Y, Nakashima Y, Takasu K, Yamabe H. Immunohistochemical localization of vitamin B12 R-binder in uterine cervical and endometrial adenocarcinomas. *Acta Hist Cyto* 1993;26:507-14.
 44. Marino M, Pinchera A, McCluskey RT, Chiovata L. Megalin in thyroid physiology and pathology. *Thyroid* 2001;11:47-56.
 45. Stupperich E, Nexø E. Effect of the cobalt-N coordination on the cobamide recognition by the human vitamin B12 binding proteins intrinsic factor, transcobalamin and haptocorrin. *Eur J Biochem* 1991;199:299-303.
 46. Fedosov SN, Berglund L, Fedosova NU, Nexø E, Petersen TE. Comparative analysis of cobalamin binding kinetics and ligand protection for intrinsic factor, transcobalamin, and haptocorrin. *J Biol Chem* 2002;277:9989-96.
 47. Fedosov SN, Fedosova NU, Krautler B, Nexø E, Petersen TE. Mechanisms of discrimination between cobalamins and their natural analogues during binding to the specific B12-transporting proteins. *Biochemistry* 2007;46:446-58.
 48. Wuerges J, Geremia S, Randaccio L. Structural study on ligand specificity of human vitamin B12 transporters. *Biochem J* 2007;403:431-40.